UNIVERSITY OF CALIFORNIA, SAN DIEGO

Immunological and Molecular Bases of Epidermal Symptoms of Atopic Dermatitis in PLCβ3$^{-/-}$ Model

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by

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The Thesis of Siavash Namiranian is approved and it is acceptable in quality and form for publication on microfilm and electronically:

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"Phospholipase C-β3 deficiency predisposes to atopic dermatitis-like skin inflammation in a mast cell-dependent manner"

ABSTRACT OF THE THESIS

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Master of Science in Biology

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Atopic dermatitis (AD) is a chronic inflammatory disease, characterized by eczematous lesion (skin inflammation with serous discharge) and excessive pruritus (itching). The early onset, relapsing nature and worsening of scratch-itch symptoms at night results in both patients' restlessness and socioeconomic burden. In addition to the prominent skin symptoms, AD patients are prone to develop allergic rhinitis (nasal allergy) and asthma, through a progressive hypersensitization process called atopic march. Moreover, the impaired skin barrier function in AD patients makes them vulnerable for infection by microbial organisms, which can further exacerbate the inflammation of the skin. Both environmental and genetic factors contribute to

development of AD. However the exact cause of this disease is still unknown. Studies aimed at identifying the causes of AD have led to the development of multiple murine models of this disease. Observations of these models have given rise to treatments that ease AD's symptoms, but the lack of a permanent cure and the high ratio of deleterious side effects to symptom suppression necessitate the search for novel pathways that can be targeted for long lasting treatment. We analyzed the epidermis of a new mouse model in which the lack of a functional gene for PLCβ3 leads to spontaneous AD. Upon challenging the skin with extracts of house dust mite, Dermatophagoides *farina* (Der f), and Staphylococcal enterotoxin B (SEB), mice exhibited early onset AD symptoms. These symptoms appeared more severe in PLCβ3$^{-/-}$ mice compared to their wild-type littermates. By using these inducible AD models, we characterized the impact of immune cells and their downstream effectors on the epidermal phenotypes of atopic dermatitis.

INTRODUCTION

Atopic dermatitis (AD) is a chronic inflammatory skin disease with a childhood onset in 85% of cases; the disease starts with flare ups in the acute phase and progresses into pruritic eczematous lesions. There are two types of AD, extrinsic and intrinsic. 80% of the cases are extrinsic AD, which is characterized with high blood immunoglobulin E (IgE) levels, triggered by an external agent. The intrinsic type has neither increased levels of blood IgE nor a known external initiator. Although most of the patients outgrow AD by adulthood, about 33% of them get mired with the relapsing symptoms of AD for the rest of their lives (5). AD lesions affect the skin of cheek and scalp, flexures, nape and dorsal limb regions. Due to the relapsing nature of the symptoms, especially the aggravation of itchiness at night, AD often causes sleep deprivation, psychological and financial burden of the patients (2, 3). In addition, studies suggest that AD patients are predisposed to develop asthma or allergic rhinitis (nasal allergy) later in life through a process known as “atopic march”, which further emphasizes the long lasting negative impact of AD on its victims (7). Studies indicate that the prevalence of AD is reaching 25% among school age children and is associated with life-style of developed countries (1, 4, and 6). It is suggested that both environmental and genetic factors play significant roles in pathogenesis of AD. Two models have been proposed to explain the triggering cause of AD. The “outside-inside” model, suggests that defects in the barrier function of skin, which allows invasion of pathogens into the epidermis, trigger the severe inflammatory responses in AD patients. On the other hand, the “inside-outside” model suggests that imbalanced immune responses followed by structural remodeling events leads to AD in the patients. It is the remodeling events that make the skin vulnerable to

invasive pathogens, which further exacerbate the symptoms of AD. Both models are supported with specific observations, some of which are discussed below.

Outside-Inside model:

The skin is the outermost protective barrier against environmental assaults. There are two basic compartments of skin, dermis and epidermis. The inner layer of skin is the dermis, which consists mostly of connective tissue and a dispersed array of fibroblasts. The outer layer of skin is called epidermis, which consists of stratified keratinocytes that form the basal, spinous, granular and corneum layers, in an innermost to outermost order. Each of these distinct layers is marked with specific protein markers; keratin 5 (K5) is predominantly expressed in the basal layer, where progenitor cells are located, K1 marks the spinous layer, where differentiated cells reside at, and loricrin designates the differentiated cells in the granular layer. The outermost layer of epidermis, known as stratum corneum (SC), has a "brick and mortar" structure (7). The brick subunits are comprised of corneocytes, which are a dead, anucleated, skeletally reinforced version of epidermal cells in granular layer. The mortar components refer to the lipid lamellae that surround the corneocytes. Lipid lamellae, which consist of cholesterol, ceramides, fatty acids and cholesterol esters, are secreted by the granular cells as they transform into corneocytes. Due to its hydrophobic

property, the lipid lamellae prevents excess dehydration and the entrance of unwanted hydrophilic substances (9, 10), as well as providing the flexibility to SC. In addition, a specific type of adhesion molecule, known as corneodesmosome, plays a critical role in maintaining the integrity of SC (19). Corneodesmosomes attach the corneocytes together in a fashion that they are referred to as metal rods passing through the bricks, in the "brick and mortar" model. During skin renewal, a group of proteases cleave these corneodesmosomes to release the corneocytes that are shed from the skin surface in a process named desquamation (13,14,15,16). Two of these proteases, which have been extensively studied, are the stratum corneum chymotryptic enzyme (SCCE) and the stratum corneum tryptic enzyme (SCTE). The proteolytic activity of these SC proteases is regulated by protease inhibitors, such as LEKTI, which help prevent the excessive desquamation and thinning of SC.

Genetic epidemiological studies on patients with inflammatory skin diseases and their healthy counterparts suggest that defects in the epidermal protein expression may contribute to the pathogenesis of inflammatory skin diseases. One of the proteins shown to be nonfunctional in most AD patients is filaggrin (21). Studies show that loss of function mutations in the filaggrin gene (FLG) impairs the barrier function of stratum corneum. Although 40% of individuals with filaggrin mutations never experience AD, the significant role of filaggrin in skin makes it impossible to deny its contribution to AD induction (35). Filaggrin, which acts as a scaffold to bundle keratin filaments together in the

granular layer of epidermis, facilitates the formation of cornified envelope, which replaces the plasma membrane in the corneocytes of the SC. The absence of filaggrin results in improper formation of the cornified envelope and reduces the assault bearing capacity of the corneocytes. In addition, deamination of filaggrin followed by proteolytic cleavage and processing of derived amino acids give rise to natural moisturizing factors (NMF) in the corneocytes. The NMF act as osmolytes to help the corneocytes retain adequate moisture, keeping them engorged. Decreased levels of NMF have been observed in AD patients (12). The lack of NMF leads to collapse of the corneocytes and gap formation in the brick-mortar structure of SC, increasing the transepidermal water loss (TEWL). Mouse models of filaggrin, called flaky tail (ft), show AD with xerosis (dry and scaly skin) symptoms, and are susceptible to allergens penetration. In addition to filaggrin-associated defects, abnormalities in the function of SC proteases and their inhibitors can cause skin barrier defects in patients with inflammatory skin diseases. One such abnormality has been identified through the observations made on patients with Netherton syndrome. In these patients, a mutation in SPINK-5 gene, which encodes LEKTI inhibitor of SC proteases, causes excessive SCCE and SCTE activation and desquamation (17, 20). Although SPINK-5 mutation was initially associated with symptoms of Netherton syndrome, further studies have found a link between this mutation and AD (18). Moreover, in transgenic mouse models of SCCE, overexpression of this protease in SC causes premature corneodesmosomal cleavage and AD with flare up symptoms. Besides genetic predispositions, microenvironmental changes in SC,

caused by external agents can have equally adverse effect on barrier function of skin. For example, excessive usage of soap and detergents raises the normally acidic pH of skin, and causes a hyperactivation of SC proteases and thinning of SC (22). Also, the rise of skin pH has an inhibitory effect on lipid lamellae production, which further abrogates SC's protective function.

Inside-Outside Model

The immune system is a network of cells, tissue and organ that protect the body against foreign invaders. It is comprised of innate and adaptive immune systems. T cells are part of the adaptive immune system and have been implicated in inflammatory diseases. Progenitor T cells can give rise to CD8+ (cytotoxic), CD4+ (helper) and T regulatory lineages. The T helper (Th) cell lineage is further divided into Th1 and Th2 types, distinguished by specific cytokines they release. The secreted cytokines determine the pro-inflammatory vs. the anti-inflammatory state of immune responses. In a normal immune response, a balance is maintained between pro-inflammatory and anti-inflammatory pathways, and disturbances to this balance have been implicated in inflammatory diseases.

AD is distinguished from other inflammatory skin diseases like psoriasis, through its characteristic immune responses, including high serum IgE levels and Th2 skewed responses in the acute phase of the disease (23, 24). Although it is not clear what triggers the initial flare-ups in AD patients, evidence suggests that food allergens and aeroallergens play a role in this process (25, 26, 27). In AD

patients, the skin lesions are infiltrated with massive numbers of dendritic cells, mast cells, eosinophils and T cells at the lesion site. Studies targeted at identifying the key players of this immune response provide important insight into etiology of AD. One of the molecules involved in the skewed immune response of AD patient is the high affinity IgE receptor (FcεRI). FcεRI is upregulated not only on mast cells, but also on antigen presenting langerhans and dendritic cells. Upon binding of allergens to IgE and subsequent recognition of Allergen-IgE complex by FcεRI, allergens are taken up by antigen presenting cells (APC). APC, which are activated by allergens, migrate to the lymph node and activate T helper cells. In AD patient this activation of T helper cells by APC is skewed by the cytokines that are released by other immune cells and histamine, released from mast cells (28). Histamine regulates dendritic cell activation of T helper cells toward Th2 by inhibiting the production of IL12 in these cells. Degranulation of mast cells, triggered through cross linking of its surface FcεRI receptors by antigens causes release of multiple cytokines, such as IL3, IL4, IL5, IL6, IL10, IL13, TNFα (tumor necrosis factor alpha) and GMCSF (granulocyte macrophage stimulating factor), in addition to histamine. Stimulation of keratinocytes by a combination of IL4 and TNFα primes these cells to release thymic stromal lymphopoietin (TSLP). This proinflammatory cytokine further promotes a Th2 oriented activation of T helper cells by acting on dendritic cells (29). Activated Th2 cells produce IL4, IL5 and IL13 cytokines. IL4 and IL13 cytokines secreted by Th2 cells drive the isotype switching of B cells and production of IgE, which further exacerbates the IgE driven inflammatory responses by increasing mast

cells survival, mediated by cell surface FcεRI receptors (31, 32). In addition, synergic effect of IL5 and GMCSF helps maintain langerhans, eosinophil, and macrophage cell infiltration in the inflamed skin and augments the inflammatory response (33). During the chronic phase of AD, Th1 cells overtake the tissue and produce interferon-γ (IFNγ) which inhibits IL4 production of Th2 cells. IFNγ stimulates keratinocytes and initiates a cellular immune response which helps protect these cells from invasion by pathogens.

New AD Model:

Almost all the knowledge on interactions of different components of the immune system in AD has been gathered through observations on mouse models of this disease. In general, there are three types of mouse models: mice, which develop AD symptoms spontaneously, or as a result of allergen stimulation of the skin, or mice in which overexpression or lack of certain gene recapitulate some of the AD symptoms. Here, we analyze the epidermal phenotype of a new model of AD. In this model, AD is induced by epicutaneous application of *Dermatophagoides farinae* (Der f) *extract* and staphylococcal enterotoxin B (SEB) to the shaved back skin of PLCβ3$^{-/-}$ mice. Der f is extracted from house dust mites, which are a known cause of allergy in AD patients. Also, staphylococcal aureus bacteria, which produce entrotoxin B, colonize the skin of 90% of AD patients (37). PLCβ3 is an isozyme member of a large family of enzymes known as phospholipase C(PLC), which hydrolyzes phosphoinositol 4,5

bisphosphate into inositol 1,4,5 triphosphate and diacylglycerol. PLC's activity drives the activation of protein kinase C and triggers the release of calcium from intracellular storages. Although previous studies on PLC have shown this enzyme to be involved in mediating the cell proliferation pathways, a recent study by Xiao *et al*. suggested an opposite function for this enzyme in hematopoietic stem cells (HSCs) (35). Using the PLCβ3$^{-/-}$ mice as its model, Xiao *et al*. demonstrated that PLCβ3 is responsible for blocking cytokine-triggered proliferation of HSCs and their descendent myeloid lineage. Cytokines induce the proliferation of HSCs and myeloid cells through multiple signaling pathways, one of which is Jak/Stat5 pathway. Activation of the Jak/Stat5 pathway results in the phosphorylation and consequent activation of STAT5. Activated STAT5 then forms a homodimer that translocates into the nucleus and functions as a transcription factor. PLCβ3 acts as a scaffold protein to direct the phosphatase activity of SHP1 toward phosphorylated STAT5. This leads to dephosphorylation of STAT5, and prevents its downstream signaling (35). Lack of PLCβ3, in the PLCβ3$^{-/-}$ mice, causes hyperproliferation of myeloid lineage, and the characteristic high mast cell content of the bone marrow and peripheral tissues. In addition, starting at 6 months after birth, these mice develop spontaneous skin lesions, which resemble AD lesions (T. Kawakami lab, unpublished). The recapitulation of many characteristics of AD symptoms primed us to utilize these mice for better understanding the immunological and molecular bases of epidermal symptoms of this disease.

MATERIAL AND METHODS

Immunofluorescence staining

Dorsal skins were fixed in 10% formaldehyde and frozen in optimal cutting temperature (OCT) compound; next, 8 μm sections were prepared from each sample. OCT was removed from each slide by incubating for two minutes in 4% formaldehyde. Residual formaldehyde was removed with three 5 min incubations in (1x) Phosphate buffered saline (PBS**)**. Sections were blocked using blocking buffer (1% bovine serum albumin, 2.5% normal goat serum, 2.5% normal donkey serum, 0.2% gelatin, 0.1% TritonX-100, dissolved in 1x PBS) for 1 hr. Primary antibodies [rabbit anti Keratin 1, chicken anti Keratin 5, rabbit anti Loricrin, rat anti E-cadherin ECCD2, rabbit anti Collagen] were diluted at 1:100 dilution in blocking buffer and incubated with the sections overnight at 4°C. After three 5 min washes in 1x PBS, secondary antibodies + Dapi were incubated with each sample for 20 min. Next, sections were washed 3x 10 min in 1x PBS and mounted using Antifade. Pictures were taken using Olympus Bx51 microscope equipped with an Olympus DP70 camera at 40X resolution.

Epidermal cell extraction and culture

Skins, taken from 2 day old pups, were incubated in 2.5U/mL Dispase II (Roche) for 1hr at 37°C to detach the dermis from epidermis. Cells were extracted from the epidermis by incubating the epidermis in 0.25% trypsin-EDTA (Gibco) for 10min and sifting the digested epidermis through a 70 micron cell strainer. Cells were cultured in 3:1 v/v Dulbecco's modified Eagle's medium (DMEM) and Ham's F-12 nutrient mixture (Gibco) at 37°C and 7% CO_2. Additives

to medium included 10% chelexed fetal bovine serum (Hyclone), 10^{-10} M cholera enterotoxin (MP Biomedicals), 36.5mM sodium bicarbonate, 5µg/ml insulin (Sigma), 0.4µg/ml hydrocortisone (Sigma), 5µg/ml transferrin (Sigma), 5µg/ml T3 (3,3',5-triiodo-L-thryonine) (Sigma**)**, 50 units/ml penicillin, 50µg/ml streptomycin (Gibco), 3.25mM L-glutamine (Gibco), and 50µM calcium chloride (Fisher Scientific). Growth of Low passage cells (up to P8) were supported by NIH 3T3 J2 fibroblasts, seeded on the plates a day before splitting the epidermal cells. To rule out the input from fibroblasts in our experiments, a 1:10 v/v dilution of fibroblast conditioned medium was added to epidermal cell medium, to supplement the growth factors required by these cells. Epidermal cells were incubated with different dilutions (1:5, 1:50, 1:500 v/v) of mast cells conditioned medium to see the effect of mast cells on inducing TSLP production in these cells. To avoid the high calcium concentration of mast cells conditioned medium having an unintended effect on our epidermal cells, these media were dialyzed against 1x PBS. Epidermal media collected under each experimental condition was tested for TSLP content, using ELISA kits. In future trials, we will spike the calcium concentration from 0.05 mM to 1.2 mM prior to our experiment to assure consistency in the epidermal cells' state.

RESULTS

Although PLCβ3$^{-/-}$ mice did not show any AD symptoms up to 6 month after birth, the majority of these mice spontaneously exhibited AD symptoms after 12 months (T. Kawakami lab, unpublished). Since both external and genetic factors are contributors to pathogenesis of AD, the external factor was added to this model by the epicutaneous application of *Dermatophagoides farinae extract* (Der f) and staphylococcal enterotoxin B (SEB) to the back skin of 5 week old PLCβ3$^{-/-}$ mice and their WT littermates, which normally do not develop AD at this age (T. Kawakami lab, unpublished). As expected, early AD symptoms appeared in both strains following the treatment. Histological analysis of the skin lesions from PLCβ3$^{-/-}$ and WT mice revealed slightly more thickening of the epidermis in PLCβ3$^{-/-}$ lesions compared to WT (T. Kawakami lab, unpublished). Due to the fact that the epidermis is the primarily affected protective barrier in AD, we compared different epidermal compartments in the lesions of WT and PLCβ3$^{-/-}$ mice to identify the differences between affected compartments in these tissues. For this purpose, we used different sets of epidermal markers to look at different layers of epidermis. Staining of epidermis with K1 (keratin 1: marker of the spinous layer) and K5 (keratin 5: marker of the basal layer) indicated a slightly higher expansion of the spinous layer, but no change in the basal layer of the PLCβ3$^{-/-}$ mice compared to WT littermates (Figure 1). Also, staining of loricrin (marker of the granular layer) indicated a decrease in the thickness of the granular layer in PLCβ3$^{-/-}$ mice lesions compared to WT counterparts (Figure 1). In addition, we analyzed the keratinocyte stress marker K6. The expression of K6

is restricted to hair follicles under normal physiological conditions but expands into the epidermis in response to variety of stress stimuli. Although we detected epidermal expression of K6 in WT and PLCβ3$^{-/-}$ lesions, there was no difference between the K6 staining. This observation rules out the possibility that the cells in PLCβ3$^{-/-}$ lesion experienced higher amounts of stress, leading to more severe phenotypes in this model (Figure 1). One of the histological signs of AD is the spongy appearance of epidermis in AD patients. The impairment of intercellular adhesions between epidermal keratinocytes causes the formation of gaps between the cells, giving the tissue a spongy appearance, known as spongiosis (41). Thus, we looked at the immunofluorescence staining of cell-cell adhesions in the epidermal samples, using an antibody against E-cadherin, the key component of adherens junctions. Staining showed no visible difference between E-cadherin expression in PLCβ3$^{-/-}$ and WT lesions. This indicates that spongiosis is not an underlying cause of more pronounced AD symptoms, observed in PLCβ3$^{-/-}$ mice (Figure 1).

A survey of immune cells recruited to the induced AD lesions of PLCβ3$^{-/-}$ and WT indicated that a number of different immune cells infiltrated these tissues (T. Kawakami lab, unpublished). In order to identify the immune cells responsible for the symptoms of the induced AD model, the double knockout strains of PLCβ3$^{-/-}$ mice, from which B cells, mast cells, or T cells were eliminated, were examined. Eliminating mast cells significantly lowered the severity of AD symptoms (T. Kawakami lab, unpublished).

To determine whether the improvement of AD symptoms after eliminating mast cell had alleviated the epidermal phenotypes of the PLCβ3$^{-/-}$ mice, we examined the previously described epidermal markers (K1, K5, Loricrin, K6, E-cad) in the skin lesions of these mice. The results show that loss of mast cells slightly reduces the epidermal thickening in PLCβ3$^{-/-}$ mice, but it completely changes the K1 and loricrin expression pattern to the pattern observed in WT lesion. Further, no change in the expression level of stress marker, K6, and adherens junction marker, E-cadherin, was observed (Figure 2).These observations indicate that mast cells are associated with the epidermal phenotype of PLCβ3$^{-/-}$ model of induced AD.

In order to characterize the impact of downstream effecter molecules on the epidermal phenotypes of PLCβ3$^{-/-}$ mice, we next looked at Thymic Stromal Lymphopoietin (TSLP). TSLP is a cytokine produced by differentiated keratinocytes in the lesions of AD patients (36). It is known to signal through STAT5 phosphorylation, but no insight exists on whether PLCβ3 is involved in its signaling pathway. Immunofluorescence staining for this cytokine showed that its expression was upregulated in the spontaneous AD lesions of PLCβ3$^{-/-}$ mice, while no expression was detected in the healthy skin regions of these mice (T. Kawakami lab, unpublished). To see the effect of eliminating TSLP on the epidermal symptoms of induced AD model, we analyzed the epidermis from the TSLP receptor knockout strain of PLCβ3$^{-/-}$ mice. These mice did not develop spontaneous AD up to 12 months after birth and AD induction in five week old litters resulted in significantly lower clinical score compared to PLCβ3$^{-/-}$

littermates (Kawakami lab., unpublished). However, our immunofluorescence results indicated that blocking TSLP slightly improves the epidermal symptoms by reducing the thickness of the spinous layer (marked by K1). No change in the thickness of granular layer (marked by loricrin) or the stress levels of the epidermal cells was detected (marked by K6) (Figure 3).

To further investigate cytokines whose elimination significantly improves the symptoms of the induced AD model, we investigated the potential role of granulocyte macrophage colony stimulating factor (GMCSF). This cytokine which is released by mast cells as well as the differentiated epidermal cells under stressful conditions has been shown to drive proliferation of epidermal cells, monocytes, and macrophages (42). We employed skin sections from GMCSF/ PLCβ3 double knockout mice to investigate the role of this cytokine in causing epidermal symptoms of the PLCβ3$^{-/-}$ induced AD model. Results indicated that the absence of this cytokine in the double knockout mice significantly improves the epidermal symptoms of induced AD by reducing the thickness of the spinous and granular layers in the epidermis of the double knockout mice (Figure 4)

FIGURES

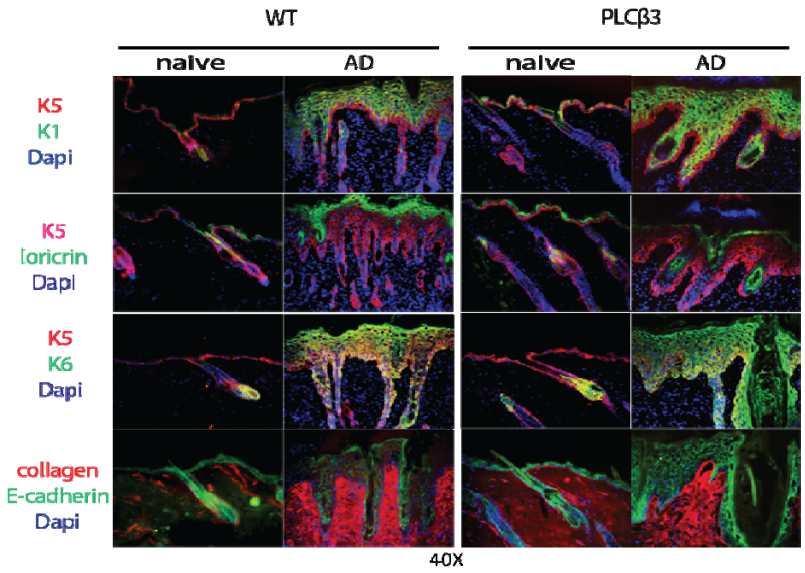

Figure 1. Induced AD model. Comparison of epidermis from healthy and lesional skin in induced AD WT and PLCβ3$^{-/-}$ models; K1(spinous layer) , K5 (basal layer), K6 (stress marker), E-cadherin (cell-cell adhesion), Collagen (dermis)

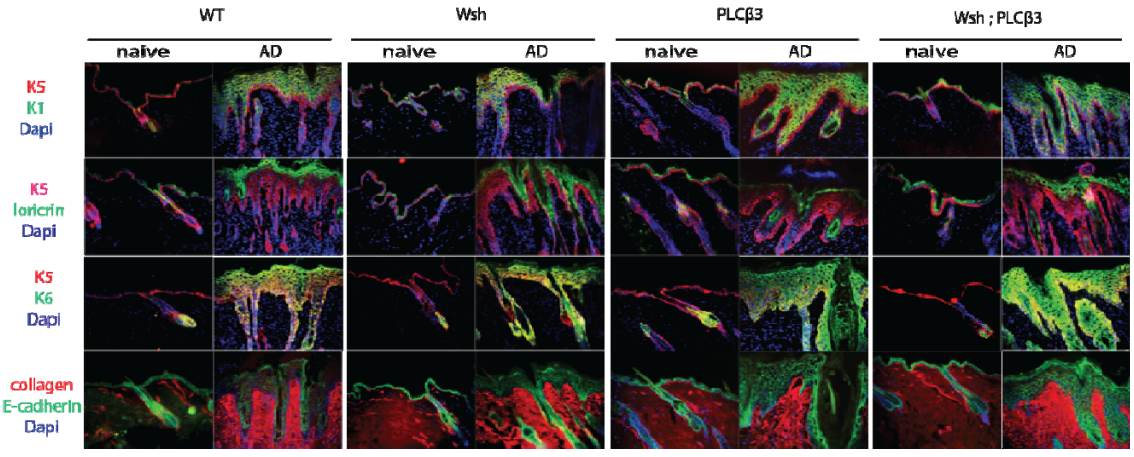

Figure 2. Effect of eliminating mast cells on the induced AD model. (A) Immunofluorescence staining of epidermal markers on the skin sections from WT, Wsh (mast cell KO), PLCβ3$^{-/-}$, and Wsh; PLCβ3$^{-/-}$ models of induced AD. K1 (spinous layer), K5 (basal layer), K6 (stress marker), E-cadherin (adhesion molecule) and Collagen (dermis)

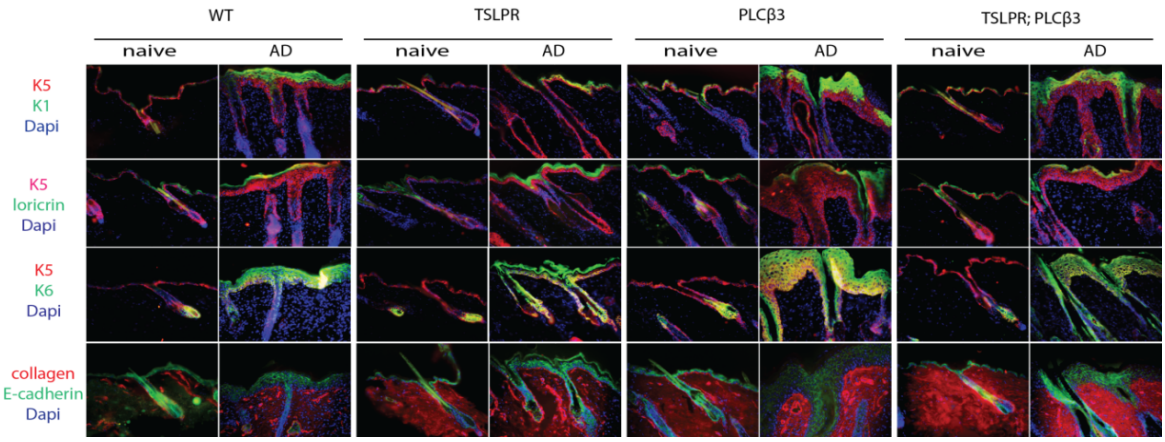

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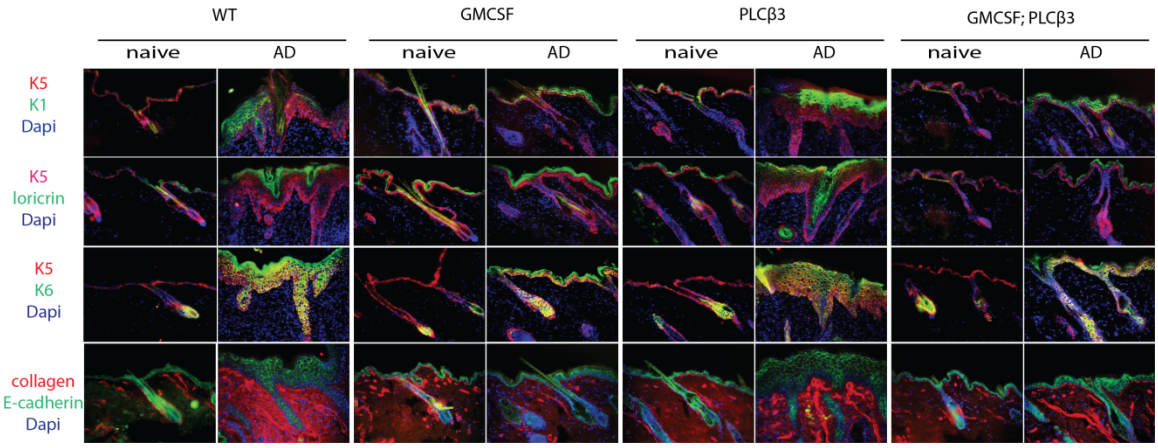
Figure 3. Role of TSLP in causing epidermal symptoms of induced AD. Immunofluorescence staining of epidermal markers on the skin sections from WT, TSLPR$^{-/-}$, PLCβ3$^{-/-}$, and PLCβ3$^{-/-}$;TSLPR$^{-/-}$ mice. K1 (spinous layer), K5 (basal layer), K6 (stress marker), E-cadherin (adhesion molecule) and Collagen (dermis).



40X

Figure 4. The role of GMCF in causing epidermal symptoms of induced AD. Immunofluorescence staining of epidermal markers on the skin sections of WT, GMCSF$^{-/-}$, PLCβ3$^{-/-}$, and PLCβ3$^{-/-}$; GMCSF$^{-/-}$ mice. K1 (spinous layer), K5 (basal layer), K6 (stress marker), E-cadherin (adhesion molecule) and Collagen (dermis)

DISCUSSION

Here we analyzed the epidermal symptoms of a new model of AD. In this model, hyperproliferation of mast cells caused by loss of PLCβ3 mediated suppression of STAT5 activity in myeloid cells is the primary cause of spontaneous AD lesion formation (T. Kawakami lab, unpublished). Furthermore, appearance of more severe symptoms in PLCβ3$^{-/-}$ mice compared to WT littermates, upon AD induction, imply that the genetic defect of PLCβ3$^{-/-}$ mice is the underlying cause of these pronounced symptoms (T. Kawakami lab, unpublished). The K1 staining of epidermis from the induced AD models indicated that the slightly greater hyperthickening of epidermis in the PLCβ3$^{-/-}$ mice, compared to WT littermates, is attributable to expansion of the differentiating cell housing spinous layer in the epidermis. Also, we noted that there was a decrease in the loricrin marker of granular layer in PLCβ3$^{-/-}$ lesion, that may be indicative of a delay in transition from the spinous to granular layer. This K1/Loricrin expression pattern was reverted to a WT AD lesion pattern in mast cell knockout strain of PLCβ3$^{-/-}$ model, indicating a possible link between mast cells and the transitioning cells of spinous layer in the epidermis. However, lack of significant improvement in epidermal symptoms of induced AD, in mast cell deficient PLCβ3$^{-/-}$ mice indicated that mast cells are not the only effecter in this immune response. Thus, mast cells play a parallel role with other immune cell types in exacerbating the symptoms of induced AD in PLCβ3$^{-/-}$ mice. Also, in correlation with studies that found high amounts of TSLP expression in the lesions of AD patients, an upregulation of TSLP in the lesions of spontaneous AD

model mice was observed (T. Kawakami lab, unpublished). Although, blocking TSLP signaling in the PLCβ3$^{-/-}$ mice completely abrogated the incidence of spontaneous AD and improved the clinical scores of the mice after AD induction (T. Kawakami lab, unpublished), the epidermal phenotypes of these double knockout mice were only slightly improved compare to PLCβ3$^{-/-}$ mice. This shows that TSLP has a redundant role as other cytokines in causing the symptoms. Similar results observed in mast cells and TSLP deficient PLCβ3$^{-/-}$ mice, regarding the incidence of spontaneous AD, suggest that there may be a possible link between TSLP producing keratinocytes and mast cells. In fact two different notions exist on interaction of mast cell and TSLP producing keratinocytes in AD. One suggests that the TSLP production in keratinocytes depends on mast cells (38), whereas the other one considers TSLP a master switch that affects many immune cells including the mast cells (40). Although, considering our results which indicate mast cells hinder the transition of spinous layer cells and the fact that differentiated keratinocytes are a major source of TSLP, support the first notion, an *in-vitro* investigation is required in order to clearly demonstrate this link.

As opposed to mast cells and TSLP, GMCSF elimination did not prevent the incidence of spontaneous AD (T. Kawakami lab, unpublished). But, it greatly improved the epidermal symptoms of the induced AD in PLCβ3$^{-/-}$ mice by reducing the thickness of both spinous and granular layers. These results indicate that GMCSF plays a more significant role in driving the epidermal phenotypes of induced AD.

As a follow up to this study we looked into interaction of mast cells and keratinocytes (epidermal cells) in an *in-vitro* model system. This allowed us to exclude the input from other immune cells. Since TSLP is known to be produced by the lesional epidermal cells and knocking out its receptor, prevented the incidence of spontaneous AD in the PLCβ3$^{-/-}$ mice, we looked at the production of this cytokine by keratinocytes upon the stimulation by mast cells conditioned medium. The results from this study are not yet conclusive. Higher TSLP production was detected for the WT rather than PLCβ3$^{-/-}$ keratinocytes, which is contradictory to the more severe lesions observed in PLCβ3$^{-/-}$ mice. But we observed higher rate of differentiation in the WT keratinocytes compared to PLCβ3$^{-/-}$ cells. Referring to the previous studies that identified differentiated keratinocytes as the source of TSLP, this may explain our results. To test this hypothesis we will repeat these experiments on keratinocytes after triggering differentiation in both PLCβ3$^{-/-}$ and WT cells to assure consistent cells state during mast cells conditioned media treatment.

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